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13. ABSTRACT ***(Maximum 200 Words)***

The HER-2/*neu* proto-oncogene is amplified and overexpressed in approximately 25% of breast cancers. Amplification and overexpression of HER-2/*neu* is correlated with poor patient prognosis, lack of responsiveness to tamoxifen treatment, responsiveness to adriamycin chemotherapy and responsiveness to Herceptin anti-HER-2/*neu* immunotherapy. In this proposal we are characterizing changes in cell adhesion related to HER-2/*neu* overexpression. We have engineered two human cell lines to overexpress HER-2/*neu* . Relative to parental control cell lines HER-2/*neu* overexpressing cell lines showed a loss of cell adhesion on selected extracellular matrix proteins, especially denatured collagen type I and vitronectin. The findings suggest that the αvß3 integrin receptor may be involved in this process since αvß3 integrin receptor mediates binding to these extracellular matrix proteins.

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Introduction

The HER-2/*neu* proto-oncogene is amplified and overexpressed in 25% of breast cancers. In animal models and in the human disease overexpression of this membrane receptor protein is considered to play a pathogenic role in the disease process. Clinically, overexpression is correlated with a shorter disease-free interval and a shorter overall survival of women with breast cancer. Overexpression is also correlated with lack of responsiveness to tamoxifen anti-hormone therapy and responsiveness to selected forms of chemotherapy. Recently, immunotherapy with monoclonal antibodies to the extracellular domain of HER-2/*neu* has been associated with both partial and complete remissions of metastatic breast cancer in 10 to 20% of breast cancers with HER-2/*neu* overexpression. Although amplification and overexpression of HER-2/*neu* is clearly important in the clinical management of women with breast cancer, relatively little is known about the molecular mechanisms by which this gene alters cell physiology to produce a malignant phenotype. Most studies of HER-2/*neu* as a transforming gene have characterized primarily tumor growth rate in culture or in xenografts, and colony formation on soft agar. None, to our knowledge, have evaluated cell adhesion to individual extracellular matrix molecules. Our primary goal in this proposal is to select this single phenotypic change associated with malignant transformation, altered cell adhesion, and characterize the molecular mechanisms contributing to this phenotype in HER-2/*neu* overexpressing cells.

Altered cell adhesion is an important characteristic of tumor cells because it is a necessary component of tumor cell anchorage-independent growth, ability to migrate, digest an extracellular matrix, and stimulate angiogenesis. Characterization of the molecular basis for alterations in tumor cell adhesion may provide a better understanding of the molecular mechanisms involved in more complex behaviors of tumor cells. An understanding of the mechanism(s) of altered cell adhesion provide the potential for intervening to restore normal cell adhesion to HER-2/*neu* overexpressing tumor cells.

Body

Although a number of studies have shown that HER-2/*neu* oncogene overexpression causes transformation of cells, relatively little is known about the mechanism (or mechanisms) by which overexpression establishes and maintains a malignant phenotype. During preliminary studies using a HER-2/*neu* -overexpressing cell line we found alterations in cell adhesion which suggested that integrins, especially αvß3, played a role in mediating cell adhesion in tumor cells. Our working hypothesis which can be stated as follows:

> HYPOTHESIS: HER-2/*neu* overexpression causes changes in cell adhesion through formation of a multimeric complex that includes the integrin receptor αvß3 and focal adhesion kinase (FAK) to cause disaggregation of integrin-mediated focal adhesions and, therefore, dissassociation from extracellular matrix proteins. A critical step in this process is proposed to be dephosphorylation of FAK by an as yet unidentified protein tyrosine phosphatase that interacts directly with HER-2/*neu*.

In order to address this hypothesis we have proposed accomplishing a series of tasks. Our progress with these tasks is summarized below.

Task 1. Characterize changes in cell adhesion associated with HER-2/*neu* overexpression relative to HER-2/*neu* low expression using individual extracellular matrix molecules (laminin, fibronectin, collagen, denatured collagen and vitronectin).

We have used assays developed primarily for the characterization of integrins to assess alterations in cell adhesion observed with HER-2/*neu* gene overexpression. Paired cell lines, overexpressing HER-2/*neu* and vector control cells (NIH-189 and NIH-3T3, HBL100 and HBL100-HER2), were grown in tissue culture medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cell adhesion assays are performed in triplicate with 1.0 x 10^5 tumor cells per well in 48-well cluster plates prepared with individual extracellular matrix proteins (human fibronectin, human vitronectin, native collagen type I, heat-denatured collagen, type I, and laminin) as described previously (1). Control wells are prepared with 1% bovine serum albumin. Tumor cells were incubated in adhesion buffer (0.5% BSA, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$) at 37°C for 30 minutes. Wells are washed three times with PBS and stained with crystal violet. Crystal violet is quantified by colorimetric analysis at 600 nm after elution with 10% acetic acid and correction for controls.

Striking differences were observed in cell adhesion to different ECM proteins in both cell lines (Figures 1 and 2). Compared with parental control NIH-3T3 cells, the HER-2/*neu* overexpressing NIH-189 cells showed a marked reduction in cellular adhesion to three ECM proteins: denatured collagen type I (5.8% of control), vitronectin (6.1% of control), and native collagen type I (37.3% of control). Cell adhesion to fibronectin (54.5% of control) and laminin (75% of control) was also reduced (Figure 1). The results indicate both a marked change in cell adhesion and a specificity of those changes related to different ECM proteins.

This year we have also spent a considerable amount of time engineering an additional cell line with HER-2/*neu* overexpression. Low expression MCF-7 human breast cancer cells were stably transfected with a HER-2/*neu* expression vector using the same Methods involved in obtaining the HBL100-HER2 overexpressing cell lines. We used fluorescence-activated cell sorting (FACS) to preliminarily identify and isolate MCF-7 transfectants with $p185^{HER\text{-}2/neu}$ overexpression. Thirty-six subclones were initially isolated and western immunoblot analysis was used to confirm that these HER2 transfectants had overexpression. Sixteen subclones have been further subcloned to ensure that each subclone is monoclonal in its origin. These subclones are currenly being expanded and will be characterized for changes in cell adhesion during the current grant year.

Cell Adhesion Assays of NIH-3T3 Cells and HER-2/*neu* Overexpressing NIH-189 Cells.

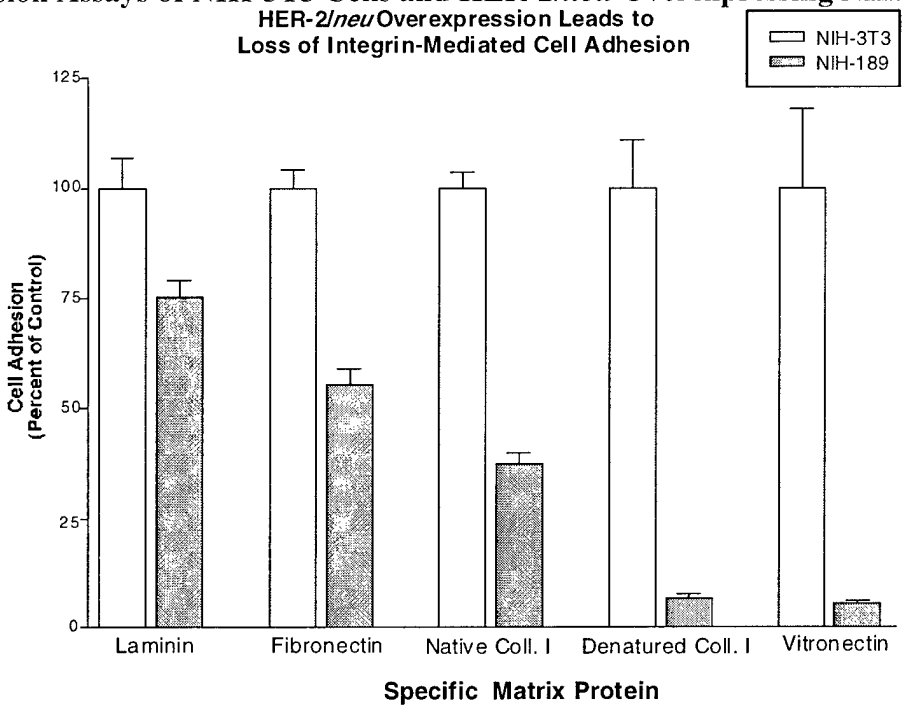

Figure 1. Assessment of cell adhesion of HER-2/*neu* -overexpressing cells (NIH/189) as a percentage of adhesion observed in control low expression cells (NIH/3T3) to various extracellular matrix proteins. Cell adhesion to denatured collagen type I (7% of control cells) and vitronectin (6% of control cells) was markedly reduced.

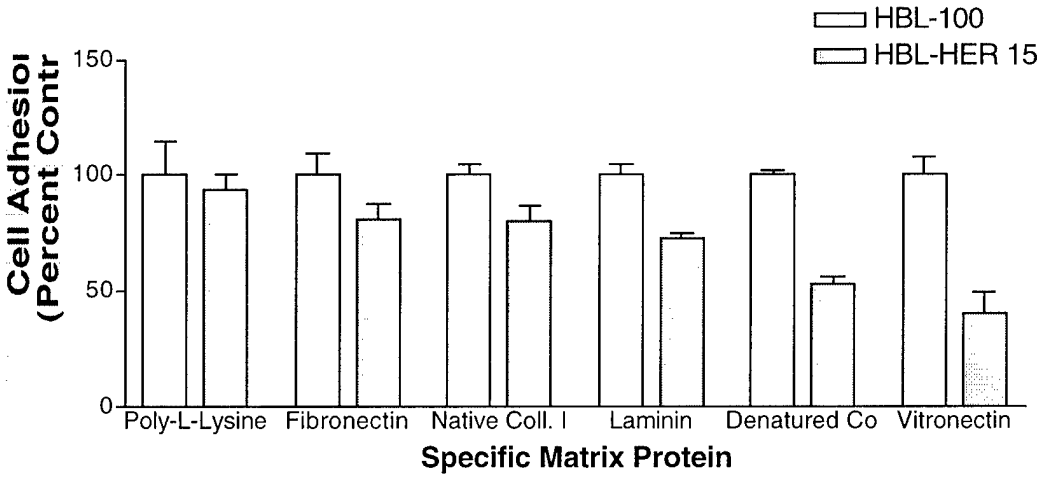

Figure 2. Assessment of cell adhesion of HER-2/*neu* -overexpressing cells (HBL100-HER2) as a percentage of adhesion observed in control low expression contron, parental cells (HBL100) to various extracellular matrix proteins. Cell adhesion to denatured collagen type I and vitronectin was markedly reduced as it was for high-expression NIH189 cells compared with low expression NIH3T3 cells.

Task 2. Determine which integrins, if any, play a role in alterations of cell adhesion observed with HER-2/*neu* overexpression.

Our preliminary observations of differences in cell adhesion to various ECM proteins, described in Task 1, indicated that αvß3 integrin receptor was almost certainly involved in altered adhesion of HER-2/*neu* overexpressing NIH-189 and HBL100-HER2 cells. The two ECM proteins which showed the most impressive alteration in adhesion, vitronectin and denatured collagen, type I, are both associated with binding by the αvß3 integrin receptor. Although other integrins (αvß1 and αvß5) can bind vitronectin, the αvß3 integrin is known as the "vitronectin receptor". In addition, the αvß3 integrin receptor is largely responsible for adhesion to denatured collagen, type I. Because the αvß3 integrin receptor appeared to be a pivotal molecule in mediating at least some of the HER-2/*neu* -associated alterations in cell adhesion, we assessed expression levels of this integrin. The simplest explanation for reduced adhesion to denatured collagen type I would have been a reduction in expression of αvß3 integrin in HER-2/*neu* overexpressing tumor cells compared with low expression parental cells. We performed western immunoblot analysis in order to test this hypothesis and were surprised to find no reduction in either αv or ß3 subunits of integrin in HER-2/*neu* overexpressing NIH-189 cells compared to NIH-3T3 cells (Figure 3).

Western Immunoblot Analysis of Integrin Subunits αv and ß3 in Low Expression NIH-3T3 Cells and HER-2/*neu* Overexpression NIH-189 Cells.

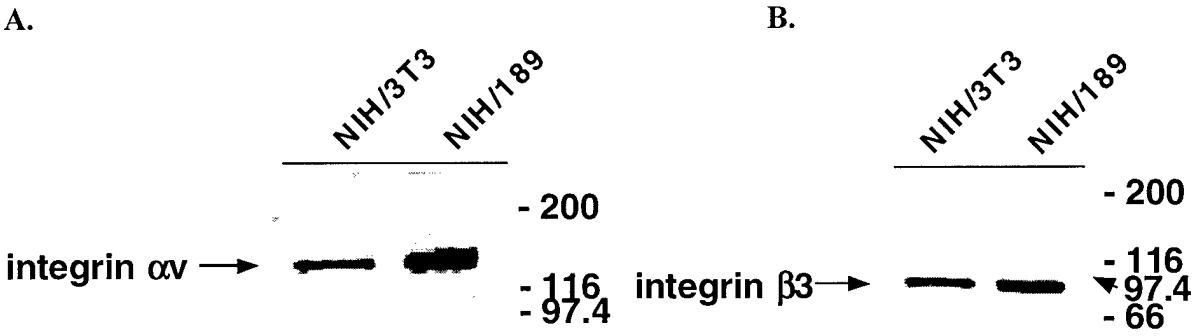

Figure 3. Western Immunoblot Analysis of Integrin Subunits αv and ß3 in Low Expression NIH-3T3 Cells and HER-2/*neu* Overexpression NIH-189 Cells. A.) Western immunoblot analysis demonstrates αv integrin both in cells with low expression (NIH-3T3) and in cells with overexpression of HER-2/*neu* (NIH-189). B.) Western immunoblot analysis demonstrates ß3 integrin both in cells with low expression (NIH-3T3) and in cells with overexpression of HER-2/*neu* (NIH-189).

Another potential source of altered cell adhesion mediated by αvß3 could be co-expression of matrix metalloproteinase type 2 (MMP-2) by the tumor cells with binding of MMP-2 at the carboxy-terminal PEX domain to αvß3 integrin receptor (2). Denatured collagen is observed in tissues during proteolytic digestion of the ECM in angiogenesis and in tumor cell invasion (1, 2). ECM degradation requires the coordinated interaction of matrix metalloproteinases, especially

MMP-2, and integrins. Since MMP-2 is bound to αvß3 integrin during ECM degradation, αvß3 could be important in other aspects of tumor growth in addition to cell adhesion (2). This possibility was investigated with western immunoblot analysis of cells grown in culture and tumors grown as xenografts in the chorio-allantoic membranes of chick embryos (1, 2) (Figure 4). Interestingly, MMP-2 was expressed by NIH-3T3 cells grown in culture and in chorio-allantoic membranes but was not expressed by HER-2/*neu* overexpressing NIH-189 cells in culture or in chorio-allantoic membranes (Figure 4). A monoclonal antibody, TV88, to the carboxyterminal PEX domain was also used in order to exclude the possibility that the PEX domain alone was associated with αvß3 integrin after activation of MMP-2 with cleavage at the PEX domain (data not shown). This was the opposite of what would have been predicted if MMP-2 or PEX associated with αvß3 to alter cell adhesion. As a result of these observations we considered the possibility that HER-2/*neu* overexpression affected alterations in cell adhesion through alterations in the signal transduction pathways of the cell.

Expression of Matrix Metalloproteinase-2 in HER-2/*neu* Overexpressing (NIH-189) and Control (NIH-3T3) Cells.

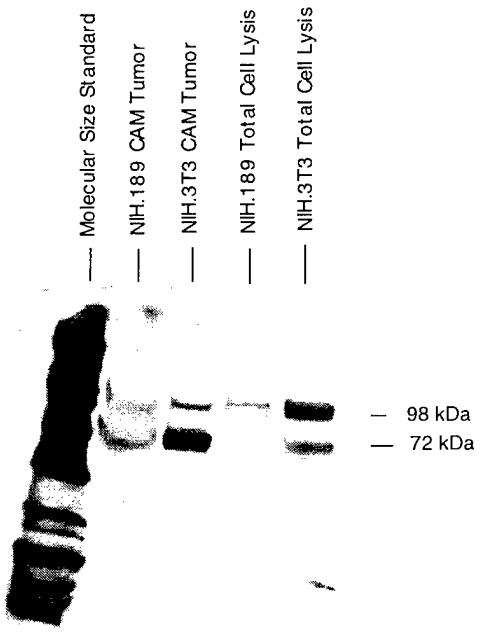

Figure 4. Matrix Metalloproteinase-2 (MMP-2), a 72 kDa protein, was expressed in $p185^{HER\text{-}2/neu}$ low expression control cells either in culture or in tumors grown as xenografts in chorio-allantoic membranes of chicks (CAM). However, substantially less (or undetectable) levels of MMP-2 expression were present in $p185^{HER\text{-}2/neu}$ high-expression NIH189 cells and NIH189 CAM xenografts.

Task 3. Assess the molecular mechanism responsible for alterations in cell adhesion of HER-2/*neu* overexpressing cells.

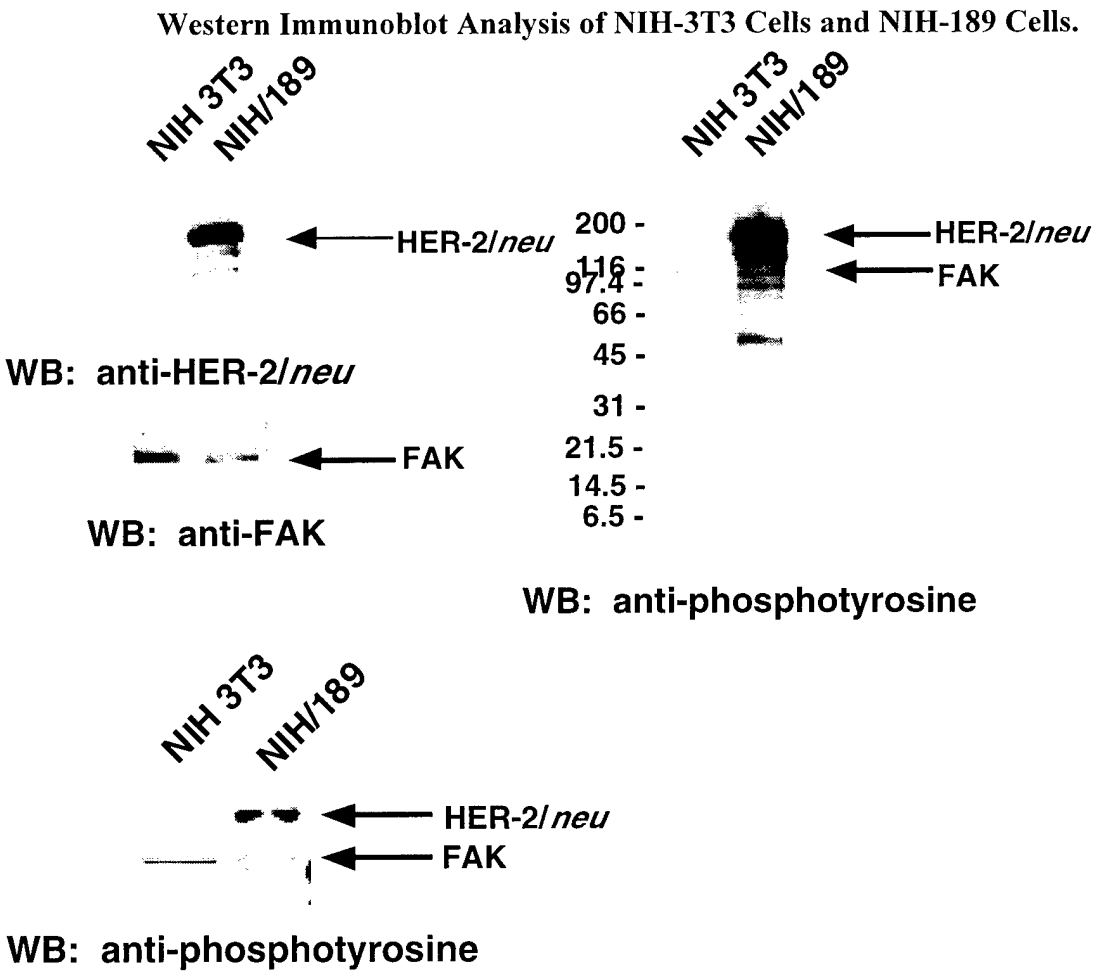

Figure 5. Western Immunoblot Analysis of NIH-3T3 Cells and NIH-189 Cells. (Upper Left Illustration.) Monoclonal 10H8 anti-HER-2/*neu* antibody demonstrated an increased level of expression of $p185^{HER\text{-}2/neu}$ membrane receptor in molecularly engineered NIH-189 cells relative to low expression parental NIH-3T3 cells. The engineered NIH-189 cells clearly showed overexpression of human $p185^{HER\text{-}2/neu}$ membrane receptor whereas parental NIH-3T3 cells did not. (Middle Left Illustration.) The same cell lines demonstrated nearly equal amounts of focal adhesion kinase (FAK) in cell lysates indicating equal loading of samples. (Upper Right.) Western immunoblot analysis of phosphotyrosine in proteins from the low expression NIH-3T3 cells and HER-2/*neu* overexpression NIH-189 cells demonstrated high levels of phosphotyrosine in several proteins from NIH-189 cells but not from NIH-3T3 cells. (Lower Left) Repeat western immunoblot analysis of phosphotyrosine in NIH-3T3 and NIH-189 on a higher percentage (7.5% SDS-PAGE) gel demonstrated better resolution of FAK from other high molecular weight proteins.

HER-2/*neu* is known to interact with other EGF receptor family members and signal transduction proteins (7); however, it is not known to interact with or regulate proteins which

control cell adhesion. Because our data clearly demonstrated a change in adhesion that was mediated by integrins, our approach is to systematically investigate the expression, activation and physical association of integrins in this functional.

Our results confirmed that HER-2/*neu* overexpression markedly up-regulated tyrosine phosphorylation of selected proteins including HER-2/*neu* (Figure 5, upper right). We considered several different proteins involved in the formation of focal adhesions as potential candidates for interactions with HER-2/*neu* to regulate the formation (or dissolution) of adhesive sites. These included proteins which mediate the association between integrins and actin filaments such as talin, vinculin, paxillin, tensin and α-actinin. Among these only vinculin showed a significant difference in either expression or phosphotyrosine activation.

Signal transduction by integrins involves activation of intracellular protein tyrosine kinases, especially the focal adhesion-associated kinase (FAK) (3). We hypothesized that activation of FAK, as a pivotal adhesion regulatory protein, may be altered by HER-2/*neu* overexpression. In order to address this possibility we investigated FAK expression and tyrosine phosphorylation of FAK in HER-2/*neu* overexpressing and control cells. We confirmed that FAK was not only expressed in these cell lines (Figures 5 and 6) but was also associated with integrin ß3 by immunoprecipitation assay (Figures 6) as described by others (1). Similarly, we addressed the potential for association between FAK and HER-2/*neu* and observed that immunoprecipitation with anti-FAK antibody also precipitated HER-2/*neu* which was demonstrated by immunoblotting of the precipitated proteins with 10H8 anti-HER-2/*neu* antibody (Figure 6). Conversely, immunoprecipitation with 10H8 anti-HER-2/*neu* antibody showed an association with FAK when the precipitates were immunoblotted with the anti-FAK antibody (Figure 6). Of particular interest was the observation that immunoprecipitation with anti-FAK antibodies followed by immunoblotting with anti-phosphotyrosine antibody demonstrated that although FAK was present in approximately equal amounts in HER-2/*neu* -overexpressing and control cell lines, only FAK from control cells demonstrated more than trace phosphorylation of tyrosine residues (Figure 6). This suggested that FAK was either not phosphorylated or that it was dephosphorylated after association with HER-2/*neu* . In order to support this as a potential mechanism for altering cell adhesion in HER-2/*neu* overexpressing cells, we treated NIH-3T3 cells grown with emodin, an inhibitor of tyrosine kinase activity known to prevent phosphorylation of c-*src* (3). c-*src* mediates integrin-related tyrosine phosphorylation of FAK in NIH-3T3 cells (3). We expected inhibition of FAK phosphorylation to alter cell adhesion in NIH-3T3 cells as we had observed in HER-2/*neu* -overexpressing NIH-189 cells lacking FAK phosphorylation. Indeed, NIH3T3 cells grown on vitronectin and laminin showed a reduction in cell adhesion similar in pattern to that observed in control NIH-3T3 cells relative to HER-2/*neu* -overexpressing cells (Figure 7).

Our current working hypothesis for the mechanism of action involves association of a protein tyrosine phosphatase (PTP) with HER-2/*neu* - FAK to remove phosphate groups from FAK. A family of such protein tyrosine phosphatases activated by tyrosine phosphorylation has been described, one of which (PTP 1D) is known to associate with HER-2/*neu* (4). Although HER-2/*neu* phosphotyrosine residues are known to be unaltered by this association, the substrate for this PTP has not been identified (4). A potential substrate for PTP 1D is FAK in the context of HER-2/*neu* overexpression. This idea will be tested with immunoprecipitation assays. Another, equally plausible phosphatase, is the PTEN tumor suppressor gene which is known to be mutated in some breast cancers and to interact with FAK and to dephosphorylate FAK (5). However, PTEN has no known association with HER-2/*neu*. Each of these proteins will be investigated as candidates to mediate an association with both HER-2/*neu* and FAK which results in loss of FAK phosphorylation.

Co-Immunoprecipitation of FAK with HER-2/*neu* and Loss of Phosphotyrosine in the Presence of HER-2/*neu*.

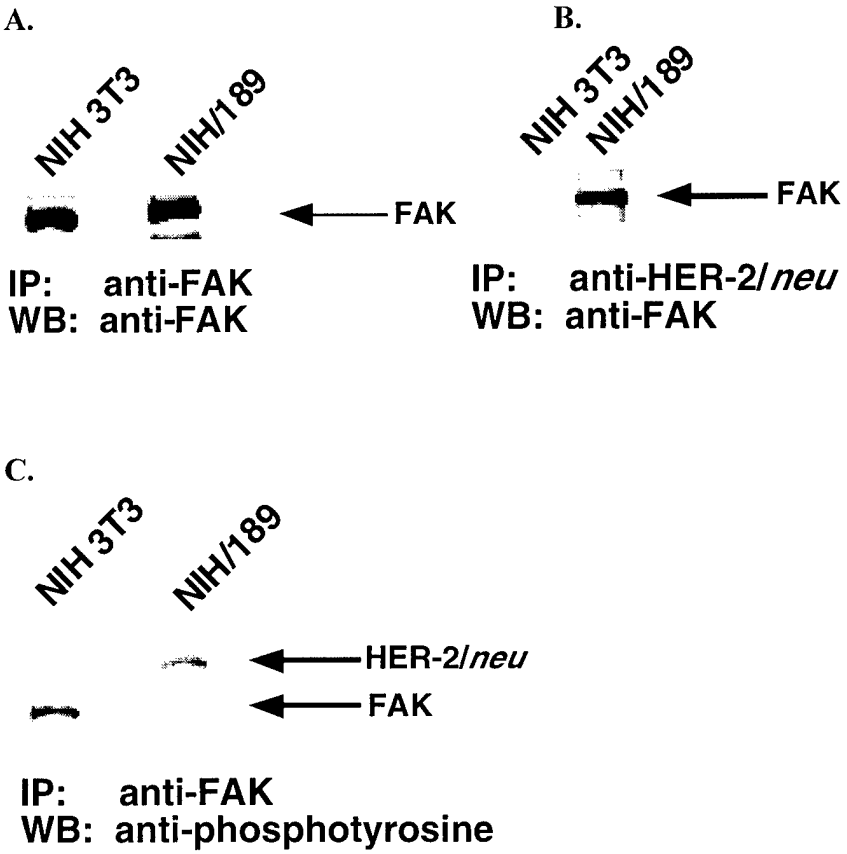

Figure 6. Co-Immunoprecipitation of FAK with HER-2/*neu* and Loss of Phosphotyrosine in the Presence of HER-2/*neu* . A.) Immunoprecipitation of FAK followed by immunoblotting with anti-FAK antibody confirms that similar amounts of FAK protein are present in low expression NIH-3T3 cells and in HER-2/*neu* overexpression NIH-189 cells. B.) Immunoprecipitation with anti-HER-2/*neu* antibody followed by immunoblotting with anti-FAK antibody demonstrates co-immunoprecipitation of these proteins in NIH-189 cells. Immunoprecipitation with the anti-FAK antibody and imminoblotting with the anti-HER-2/*neu* antibody yielded similar results (not illustrated). C.) Immunoprecipitation with anti-FAK antibody followed by immunoblotting with anti-phosphotyrosine antibody demonstrates phosphorylation of FAK in NIH-3T3 cells but not in HER-2/*neu* overexpressing NIH-189 cells even though similar amounts of the FAK protein are present in both cell lines (see 6A above).

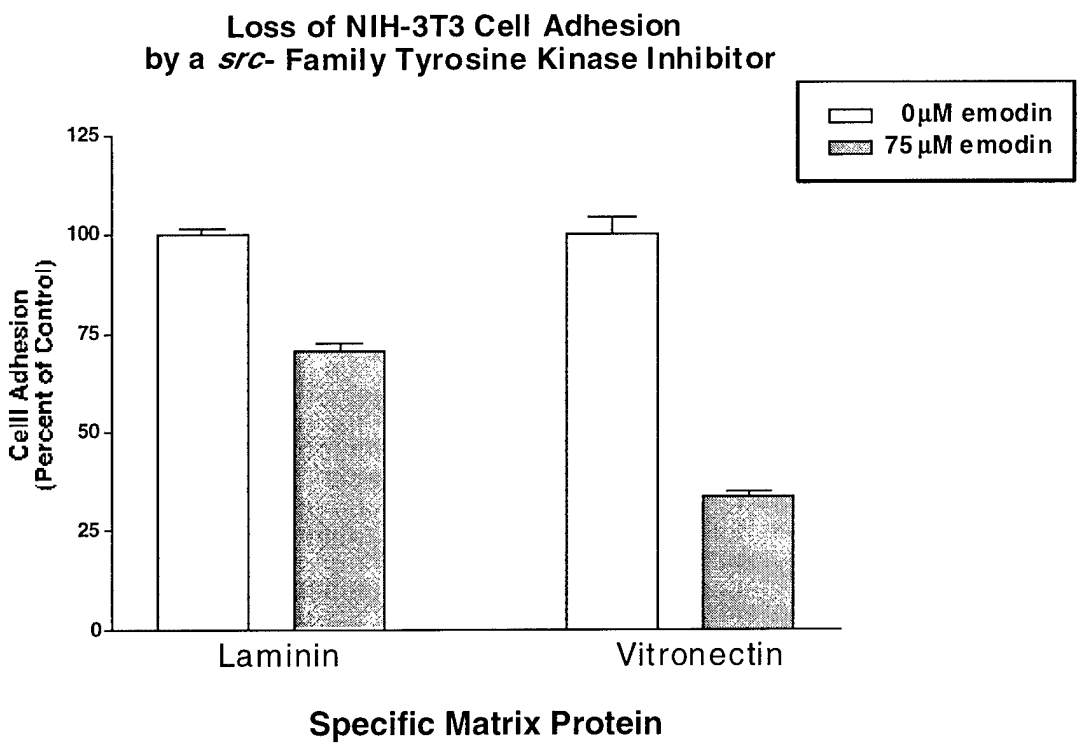

Figure 7. Loss of cell adhesion in control NIH-3T3 cells following treatment with emodin, an inhibitor of c-*src* tyrosine kinase activity. The focal adhesion kinase (FAK) is a primary target protein for c-*src* tyrosine kinase activity. Emodin, therefore, inhibits FAK phosphorylation mimicing the preliminary results observed in HER-2/*neu* overexpression cell line (NIH-189).

Key Research Accomplishments

The research accomplishments described in the Body of this Progress Report can be summarized as follows:

1. Two human breast cell lines (HBL100 and MCF-7) have been stably transfected with a HER-2/*neu* expression vector to produce engineered cell lines which differ from their parental control cell lines only with regard to the level of HER-2/*neu* expression.

2. Using these cell lines we have shown that HER-2/*neu* overexpression is associated with a loss of cell adhesion to specific extracellular matrix proteins, especially denatured collagen type I and vitronectin.

3. Although the observed pattern of altered cell adhesion strongly implicates the αvß3 integrin receptor as a mediator of the reduced cell adhesion, no loss in expression of αvß3 integrin receptor was observed.

4. The focal adhesion kinase (FAK) was not phosphorylated in HER-2/*neu* – overexpressing cell lines.

5. Treatment of control, low-expression NIH-3T3 cells with emodin, an inhibitor of c-*src* tyrosine kinase activity, resulted in a loss of cell adhesion to vitronectin and denatured collagen type I similar to that observed in HER-2/*neu* –overexpression cells. Since the focal adhesion kinase (FAK) is a primary target protein for c-*src* tyrosine kinase activity, the inhibition of FAK phosphorylation by emodin mimics the results observed in HER-2/*neu* overexpression cell lines.

Reportable Outcomes

Development of a stably transfected human breast epithelial cell line with HER-2/*neu* overexpression (HBL100-HER2) and a stably transfected human breast cancer cell line with HER-2/*neu* overexpression (MCF7-HER2).

Conclusions

HER-2/*neu* overexpression is associated with loss of cell adhesion to specific extracellular matrix proteins. The changes in cell adhesion implicate the integrin alpha-v, beta-3 receptor as a particpant in mediating this process. Changes in cell adhesion are an important part of the process by which a tumor cell migrates or becomes metastatic to other sites in the body.

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